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The tissue inhibitors of metalloproteinases (TIMPs) are specific inhibitors of MMP enzymatic activity. However, TIMP-2 can promote the activation of pro-MMP-2 by MT1-MMP. This process is mediated by the formation of a complex between MT1-MMP, TIMP-2, and pro-MMP-2. Binding of TIMP-2 to active MT1-MMP also inhibits the autocatalytic turnover of MT1-MMP on the cell surface. Thus, under certain conditions, TIMP-2 is a positive regulator of MMP activity. TIMP-4, a close homologue of TIMP-2 also binds to pro-MMP-2 and can potentially participate in pro-MMP-2 activation. We coexpressed MT1-MMP with TIMP-4 and investigated its ability to support pro-MMP-2 activation. TIMP-4, unlike TIMP-2, does not promote pro-MMP-2 activation by MT1-MMP. However, TIMP-4 binds to MT1-MMP inhibiting its autocatalytic processing. When coexpressed with TIMP-2, TIMP-4 competitively reduced pro-MMP-2 activation by MT1-MMP. A balance between TIMP-2 and TIMP-4 may be a critical factor in determining the degradative potential of cells in normal and pathological conditions.

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Introduction:

For my thesis research, I have been investigating the regulation of two members of the Matrix Metalloproteinase (MMPs) family, MMP-2 and MT1-MMP and the role of their natural inhibitors, namely, Tissue Inhibitor of Matrix Metalloproteinases (TIMPs), a family currently comprising four members. Since these enzymes are produced by fibroblasts, I tried to study their regulation in fibroblasts isolated from breast tissue. However, the manipulation of the fibroblasts turned to be very elusive. This difficulty let us to choose a vaccinia expression system to study the regulation of these matrix metalloproteinases in an eukaryotic system, which provides us with a more controllable system. We also modified **Task 1** on the proposal that states :"Parallel studies to determine the processing of MT1-MMP and the role of TIMP-2 in MMP-2 activation will be performed in a vaccinia expression system in which mammalian cells are infected and transfected with full length human MT1-MMP cDNA" to extend our studies not only on the role of TIMP-2 but on yet another inhibitor, TIMP-4 which functionally is similar to TIMP-2.

My project has dealt with a key aspect of breast cancer metastasis and had involved the use of a variety of techniques including cell biology and biochemistry in a relevant experimental model.

Body:

The activation of the zymogenic form of MMP-2 (pro-MMP-2) has been shown to be accomplished by the membrane-tethered MT1-MMP (MMP-14) (1,2), which hydrolyses the Asn^{37}-Leu^{38} peptide bond in the prodomain of pro-MMP-2 (3). To facilitate the association of the prodomain of pro-MMP-2 with the active site of MT1-MMP, pro-MMP-2 must be positioned in close association with MT1-MMP. To achieve this, TIMP-2 acts as a molecular link between pro-MMP-2 and MT1-MMP (1). It has been shown that the NH_2-terminal region of TIMP-2 binds to the active site of an active MT1-MMP on the cell surface generating a pro-MMP-2 "receptor" (4). In turn, the COOH-terminal region of TIMP-2 binds to the COOH-terminal domain of pro-MMP-2, also known as the hemopexin-like domain (HLD) (1,4-7). This trimeric MT1-MMP/TIMP-2/pro-MMP-2 complex permits the association of pro-MMP-2 to the cell surface, which eventually facilitates its activation by a neighboring TMP-2-free MT1-MMP. Under these conditions, TIMP-2 acts as a positive regulator of pro-MMP-2 activation (8). We have recently shown that in addition to its ability to form the pro-MMP-2 "receptor", TIMP-2 can also regulate the nature of MT1-MMP forms present in the cells by its ability to inhibit MT1-MMP activity (5). This effect is due to the TIMP-2 inhibition of the autocatalytic turnover of MT1-MMP on the cell surface, which may represent a natural mechanism of clearance of active MT1-MMP from the cell surface once the enzyme has fulfilled its pericellular proteolytic function. As a consequence of the inhibition of autocatalytic degradation, *de novo* synthesis and membrane incorporation of new MT1-MMP molecules, TIMP-2 binding to active MT1-MMP results in accumulation of active MT1-MMP on the cell surface (5). These dual effects of TIMP-2 (ternary complex formation and inhibition of MT1-MMP autocatalytic turnover) contribute to the overall effects of MT1-MMP on the cell surface: activation of pro-MMP-2 and direct pericellular proteolysis (9,10).

TIMP-2 belongs to a family of four TIMP inhibitors, which presently includes TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (11). Studies on TIMP-MMP interactions have shown that TIMP-4, like TIMP-2 is also capable of forming a complex with pro-MMP-2, which is mediated by binding of the inhibitor to the HLD of the enzyme (12). Thus, functionally, TIMP-4 is similar to TIMP-2. In addition, sequence analyses revealed a 70% identity between TIMP-2 and TIMP-4 (11,13). Based on these observations, we asked whether TIMP-4, like TIMP-2, could also promote pro-MMP-2 activation by MT1-MMP. To answer this question, we expressed human TIMP-4 in a vaccinia expression system (14,15) and tested its ability to promote pro-MMP-2 activation and inhibition of MT1-MMP autocatalysis in mammalian cells expressing MT1-MMP.

Purpose: The goal of this second phase was to study the regulation of MT1-MMP and MMP-2 and the role of a particular inhibitor, namely TIMP-4 in this process. Since these matrix metalloproteinases are overexpressed in many cancers, including breast cancer, it is relevant to study the role of TIMPs in the processing of MT1-MMP and MMP-2 activation. I hypothesized that TIMP-4 will affect the processing of MT1-MMP and its activation of pro-MMP-2. The purpose of these studies is to examine in detail the regulatory mechanisms underlying the processing of MT1-MMP and its activation of pro-MMP-2 to understand how these processes contribute to the degradation of ECM during cancer metastasis.

Objective: To investigate the effect of TIMP-4 on the processing of MT1-MMP and pro-MMP-2 activation .

Key Research Accomplishments:

- A cellular approach designed to express MT1-MMP in the absence or presence of TIMP-2 and/or TIMP-4 revealed a unique interaction between MT1-MMP and TIMP-4.
- We showed that TIMP-4 regulates the amount of active MT1-MMP (57 kDa) on the cell surface.
- In the absence of TIMP-4, MT1-MMP undergoes autocatalysis to a 44-kDa form, which we have previously demonstrated lacks the entire catalytic domain.
- TIMP-4 failed to promote activation of pro-MMP-2 by MT1-MMP.

List of Reportable Outcomes:

- The results of this research were published in Biochemical and Biophysical Research Communications **281**, 126-130, 2001 (**Reprint Attached**).

- Presentation of a poster in the VIIIth International Conference of the Metastasis Research Society held in London, England in September, 2000.
  Co-expression of TIMP-4 with MT1-MMP cannot promote pro-MMP-2 activation. Differential roles of TIMPs in regulation of pericellular proteolysis.
  *Sonia Hernandez-Barrantes[1], Yoichiro Shimura[1], Paul D. Soloway[2] and Rafael Fridman[1].
  [1]Department of Pathology, Wayne State University, Detroit, MI, USA, and [2]Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, NY, USA.*

- Presentation of a poster discussion session in the 92nd American Association for Cancer Research (AACR) held in New Orleans, Louisiana in March, 2001. This worked was honored with an AACR-AstraZeneca Scholar-in-Training Award (**Award Letter Attached**).
  Differential roles of TIMP-2 and TIMP-4 in pro-MMP-2 Activation by MT1-MMP. Potential implication for Pericellular Proteolysis in Malignant Processes.

<u>Conclusions:</u>

These studies established that like TIMP-2, TIMP-4 binds to MT1-MMP and inhibits MT1-MMP autocatalytic turnover. In addition, we showed the inability of TIMP-4 to promote pro-MMP-2 activation by MT1-MMP and demonstrated that this effect can only be mediated by TIMP-2 in spite of the ability of both inhibitors to form a non-covalent complex with pro-MMP-2 through its hemopexin-like domain. Although, these results suggest that the lack of pro-MMP-2 activation in the presence of TIMP-4 is due to the inability of this inhibitor to generate a ternary complex with MT1-MMP and pro-

MMP-2. Thus, while TIMP-4 binds to active MT1-MMP, the TIMP-4/MT1-MMP complex cannot act as a receptor for pro-MMP-2 and therefore activation does not ensue. Furthermore, our studies suggested that TIMP-4 competes for the binding of TIMP-2 to MT1-MMP and prevents its ability to support pro-MMP-2 activation. These observations and the fact that the expression of TIMP-4 has been shown to overlap with that of TIMP-2 in various instances suggest that a balance of these inhibitors may alter the net activity of MMP-2 with TIMP-2, under certain conditions, promoting MMP-2 and MT1-MMP-dependent proteolysis and TIMP-4 acting as a general MMP inhibitor. In conclusion, the results of this study demonstrate a differential role for members of the TIMP family in the inhibition of MT1-MMP activity and in MT1-MMP-dependent pro-MMP-2 activation.

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Appendices:

- American Association for Cancer Research Award Letter

- Reprint Attached. Biochemical and Biophysical Research Communications **281**, 126-130 (2001)

AACR
American Association
for Cancer Research

April 16, 2001

Sonia Hernandez-Barrantes
Wayne State University
Detroit, MI 48201
USA

Dear Dr. Hernandez-Barrantes:

On behalf of the American Association for Cancer Research, I congratulate you on being named an AACR-AstraZeneca Scholar-in-Training.

We are honored that AstraZeneca has continued their dedication to the development of early career scientists. Through the AstraZeneca Scholars Program, 13 young investigators were recognized for research of the highest scientific merit and were enabled to attend the AACR Annual Meeting in New Orleans. I hope that you took advantage of the many opportunities to discuss your work with leading investigators in your field and to form collaborations, mentorships, and friendships that will advance your career in cancer research.

We ask that you send a letter to the AACR offices about your experience at the AACR Annual Meeting and acknowledging AstraZeneca's outstanding commitment to early-career scientists. Please send your letter to:

> American Association for Cancer Research
> Attn: Sheri Ozard, Program Coordinator
> Public Ledger Building, Suite 826
> 150 S. Independence Mall West
> Philadelphia, PA 19106-3484

AACR will forward these letters to AstraZeneca in one package to demonstrate the effect of this program and to encourage their continued support of AACR and early-career scientists. If you have any questions, please feel free to contact me via email at wolodzko@aacr.org.

Sincerely yours,

Victoria A.M. Wolodzko
Manager, Program Administration

cc: Dr. Margaret Foti, Chief Executive Officer

Differential Roles of TIMP-4 and TIMP-2 in Pro-MMP-2 Activation by MT1-MMP

Sonia Hernandez-Barrantes,* Yoichiro Shimura,* Paul D. Soloway,†
QingXiang Amy Sang,‡ and Rafael Fridman*,1

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of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York 14263; and
‡Department of Chemistry, Biochemistry Division, Florida State University, Tallahassee, Florida 32306

The tissue inhibitors of metalloproteinases (TIMPs) are specific inhibitors of MMP enzymatic activity. However, TIMP-2 can promote the activation of pro-MMP-2 by MT1-MMP. This process is mediated by the formation of a complex between MT1-MMP, TIMP-2, and pro-MMP-2. Binding of TIMP-2 to active MT1-MMP also inhibits the autocatalytic turnover of MT1-MMP on the cell surface. Thus, under certain conditions, TIMP-2 is a positive regulator of MMP activity. TIMP-4, a close homologue of TIMP-2 also binds to pro-MMP-2 and can potentially participate in pro-MMP-2 activation. We coexpressed MT1-MMP with TIMP-4 and investigated its ability to support pro-MMP-2 activation. TIMP-4, unlike TIMP-2, does not promote pro-MMP-2 activation by MT1-MMP. However, TIMP-4 binds to MT1-MMP inhibiting its autocatalytic processing. When coexpressed with TIMP-2, TIMP-4 competitively reduced pro-MMP-2 activation by MT1-MMP. A balance between TIMP-2 and TIMP-4 may be a critical factor in determining the degradative potential of cells in normal and pathological conditions.   © 2001 Academic Press

Key Words: matrix metalloproteinases; TIMP; proteases; membrane proteins; cell surface.

The activation of the zymogenic form of MMP-2 (pro-MMP-2) has been shown to be accomplished by the membrane-tethered MT1-MMP (MMP-14) (1, 2), which hydrolyses the Asn^{37}-Leu^{38} peptide bond in the prodomain of pro-MMP-2 (3). To facilitate the association of the prodomain of pro-MMP-2 with the active site of MT1-MMP, pro-MMP-2 must be positioned in close association with MT1-MMP. To achieve this, TIMP-2 acts as a molecular link between pro-MMP-2 and MT1-

MMP (1). It has been shown that the NH_2-terminal region of TIMP-2 binds to the active site of an active MT1-MMP on the cell surface generating a pro-MMP-2 "receptor" (4). In turn, the COOH-terminal region of TIMP-2 binds to the COOH-terminal domain of pro-MMP-2, also known as the hemopexin-like domain (HLD) (1, 4–8). This trimeric MT1-MMP/TIMP-2/pro-MMP-2 complex permits the association of pro-MMP-2 to the cell surface, which eventually facilitates its activation by a neighboring TMP-2-free MT1-MMP. Under these conditions, TIMP-2 acts as a positive regulator of pro-MMP-2 activation (9). We have recently shown that in addition to its ability to form the pro-MMP-2 receptor, TIMP-2 can also regulate the nature of MT1-MMP forms present in the cells by its ability to inhibit MT1-MMP activity (6). This effect is due to the TIMP-2 inhibition of the autocatalytic turnover of MT1-MMP on the cell surface, which may represent a natural mechanism of clearance of active MT1-MMP from the cell surface once the enzyme has fulfilled its pericellular proteolytic function. As a consequence of the inhibition of autocatalytic degradation, *de novo* synthesis and membrane incorporation of new MT1-MMP molecules, TIMP-2 binding to active MT1-MMP results in accumulation of active MT1-MMP on the cell surface (6). These dual effects of TIMP-2 (ternary complex formation and inhibition of MT1-MMP autocatalytic turnover) contribute to the overall effects of MT1-MMP on the cell surface: activation of pro-MMP-2 and direct pericellular proteolysis (10, 11).

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observations, we asked whether TIMP-4, like TIMP-2, could also promote pro-MMP-2 activation by MT1-MMP. To answer this question, we expressed human TIMP-4 in a vaccinia expression system (15, 16) and tested its ability to promote pro-MMP-2 activation and inhibition of MT1-MMP autocatalysis in mammalian cells expressing MT1-MMP. Our studies demonstrate that unlike TIMP-2, TIMP-4 cannot promote MT1-MMP dependent pro-MMP-2 activation but can inhibit MT1-MMP autocatalytic degradation. Furthermore, if co-expressed with TIMP-2, TIMP-4 reduces the rate of pro-MMP-2 activation induced by TIMP-2.

MATERIALS AND METHODS

Cells. Nonmalignant monkey kidney epithelial BS-C-1 (CCL-26) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Immortalized homozygous *Timp2* (−/−) mutant mouse fibroblasts were isolated from *Timp2* deficient mice and immortalized by retroviral infection as described (7, 17, 18) and maintained in DMEM supplemented with 10% FBS and antibiotics. All tissue culture reagents were purchased from Gibco BRL (Grand Island, NY).

Vaccinia virus and construction of expression vectors. The generation of the vaccinia expression vector pTF7EMCV-1 (pTF7) containing the T7 RNA promoter and the production of vTF7-3, a recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase, have been described by Fuerst *et al.* (16). The generation of the pTF7-T2 vector expressing human TIMP-2 (15) and pTF7-MT1 expressing human MT1-MMP (6) has been described. The human full-length TIMP-4 cDNA (14), a generous gift from Dr. Y. E. Shi (Albert Einstein College of Medicine, New Hyde Park, NY 11042), was amplified by the polymerase chain reaction (PCR) using the following oligonucleotide primers: 5'-CATTCCATGGCACCTGGGAGCCCT-3' and 5'-CTTGGATCCCTAGGGCTGAACGATGTCAAC-3' containing the NcoI and BamHI restriction sites, respectively. The amplified TIMP-4 fragment was isolated and cloned into pTF7 vector (16) to generate the pTF7-T4 plasmid. The DNA sequence of the TIMP-4 PCR fragment was verified by sequencing of both strands directly from the pTF7-T4 vector using an ABI377A DNA sequencer.

Expression of recombinant proteins by infection–transfection. Monkey kidney BS-C-1 or *Timp2* (−/−) mutant cells in 6-well plates were infected with 30 plaque-forming units (pfu)/cell of the vTF-3 virus in DMEM containing 2.5% FBS (15). Thirty minutes postinfection, the media were aspirated and the infected cells were cotransfected with a mixture of either pTF7-MT1 and pTF7-T4 plasmids (04 μg/ml each) or pTF7-MT1 and pTF7-T2 plasmids (04 μg/ml each) to coexpress MT1-MMP with each inhibitor using Effectene (Qiagen, Valencia, CA), as described by the manufacturer. In some experiments, MT1-MMP was coexpressed with both TIMP-4 and TIMP-2 by transfecting a mixture of the three plasmids (04 μg/ml each). As controls, the infected cells were transfected with pTF7-MT1, pTF7-T4, pTF7-T2 or the empty pFT7EMCV-1 expression vectors alone. An additional control included cells infected with vTF-3 virus but nontransfected. Four h post-transfection, the media were aspirated and replaced with 1 ml/well of OPTI-MEM (Gibco BRL).

Activation of pro-MMP-2. Eighteen hours following the infection/transfection procedure, the cells received 3 nM/well of purified recombinant pro-MMP-2, purified to homogeneity as previously described (15), followed by a 4-h incubation at 37°C. The media were collected and clarified by a brief centrifugation (13,000g, 15 min, 4°C) and the cells were solubilized in 100 μl/well of cold lysis buffer (25

mM Tris–HCl [pH 7.5], 1% IGEPAL CA-630, 100 mM NaCl, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM benzamidine, and 1 mM PMSF) and centrifuged (13,000g) for 15 min at 4°C. Samples of the lysates (10 μl) were mixed with 4X Laemmli sample buffer (19) without reducing agents and without heating and subjected to gelatin zymography, as previously described (20).

Immunoprecipitation of TIMP-2 and TIMP-4. BS-C-1 cells Infected-transfected to express TIMP-2 or TIMP-4 with or without MT1-MMP, in 6-well plates, were metabolically labeled for 4 h at 37°C with 100 μCi/ml of 35S-methionine in 1 ml/well of DMEM without methionine supplemented with 1% dialyzed FBS. The media were collected, clarified by a brief centrifugation and an aliquot incubated (16 h, 4°C) with either 5 μg of anti-TIMP-2 CA-101 monoclonal antibody or a rabbit polyclonal antibody to TIMP-4 (21) followed by the addition of 30 μl of Protein G-Sepharose beads for an additional 3-h incubation at 4°C. After recovering the beads by a brief centrifugation, the beads were washed (5 times) with cold 50 mM Tris–HCl pH 7.5 containing 150 mM NaCl, 0.1% NP-40, and 10% glycerol and resuspended in 15 μl Laemmli sample buffer with β-mercaptoethanol followed by boiling (5 min). The immunoprecipitates were resolved by 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Detection of radiolabeled proteins was performed by autoradiography.

Immunoblot analysis of MT1-MMP forms. Infected BS-C-1 cells in 6-well plates, as described above, were lysed in cold lysis buffer. The lysates were mixed with 4× Laemmli sample buffer with β-mercaptoethanol and then resolved by 10% SDS–PAGE followed by transfer to a nitrocellulose membrane as described (20). Detection of MT1-MMP and TIMP-4 were carried out using a polyclonal antibody to human MT1-MMP (pAb 437) (22) and a polyclonal antibody to human TIMP-4 (21), respectively. Detection of the antigen-antibody complex was performed using the SuperSignal Enhanced Chemiluminescence (ECL) system (Pierce, Rockford, IL), according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Our previous studies demonstrated that co-expression of human MT1-MMP with TIMP-2 in a vaccinia expression system resulted in enhanced activation of pro-MMP-2 (6). Activation of pro-MMP-2 by MT1-MMP was TIMP-2-dependent since no activation was observed in cells devoid of TIMP-2 (*Timp2* mutant cells) (7). Furthermore, TIMP-2 presence induced the accumulation of the 57-kDa form of MT1-MMP, which represents the active enzyme starting at Y^{112}. Concomitantly with the accumulation of the 57-kDa form there was a reduction of the 44−40-kDa autocatalytic product of MT1-MMP in the cell lysates (7). To examine the effects of TIMP-4 on pro-MMP-2 activation, the full-length human TIMP-4 cDNA was cloned into the pTF7EMCV-1 vaccinia expression vector (16) and expressed in BS-C-1 cells with or without MT1-MMP using the infection-transfection procedure. For comparison, the cells were infected-transfected to co-express TIMP-2 and MT1-MMP. Analysis of TIMP-4 and TIMP-2 expression was monitored by immunoprecipitation of the inhibitors from the media of 35S-labeled cells. As shown in Fig. 1, both TIMP-2 (Fig. 1, lane 2) and TIMP-4 (Fig. 1, lane 4) were detected in the media when expressed without MT1-MMP. In contrast, co-expression of the inhibitors with MT1-MMP

Early studies showed a restricted pattern of expression of TIMP-4 in human and mouse tissues with preponderant expression in heart tissue (14, 23). However, recent studies indicate a broader tissue expression of TIMP-4 in normal tissues (24–26) and enhanced expression in pathological conditions (27–31). In various instances, the expression of TIMP-4 has been shown to overlap with that of TIMP-2 (27–29, 31). TIMP-4 has also been shown to inhibit tumor growth and metastasis of a human breast cancer cell line (32). However, its expression in human cancer tissues has not been examined in detail. Although the precise role of each TIMP in normal and pathological conditions remains to be further evaluated, the ability of TIMP-4 to inhibit the effect of TIMP-2 on pro-MMP-2 activation suggests that a balance of these inhibitors may alter the net activity of MMP-2 with TIMP-2, under certain conditions, promoting MMP-2- and MT1-MMP-dependent proteolysis and TIMP-4 acting as a general MMP inhibitor.

Presently, no information exists in regards to the molecular interactions of TIMP-4 with MT1-MMP. The crystal structures of TIMP-2 and TIMP-1 show that TIMP-2 has an insertion at Asp^{30}-Arg^{42} that extends the beta-sheets to interact with the catalytic domain of MT1-MMP. In addition, Tyr^{36} on TIMP-2 has hydrophobic interactions with the catalytic domain of MT1-MMP, fitting into a hydrophobic pocket above the active site groove near the S2/S3 site (5). These features, which are absent in TIMP-1, may partly explain the preferred binding of TIMP-2 to MT1-MMP (3, 5, 6). Energy-minimized computer modeling of the TIMP-4 structure based on the x-ray crystal structure of TIMP-2 (5) shows that TIMP-4 (12, 14), as opposed to TIMP-1, possesses a similar insertion at Val^{29}-Met^{41} that could potentially enhance interactions with the catalytic domain of MT1-MMP (Drs. Mobashery and Kotra, Dept. of Chemistry, Wayne State University, Personal communication) and inhibit MT1-MMP activity. We have previously shown that TIMP-2 induces accumulation of the 57-kDa active form of MT1-MMP (6). This effect is a direct consequence of the inhibitory action of TIMP-2 on MT1-MMP activity, which inhibits the autocatalytic turnover of active MT1-MMP into the inactive forms of 44–40 kDa. The 44-kDa form possesses an N-terminus starting at G^{285} and its formation involves the deletion of the entire catalytic domain of MT1-MMP (6). Accumulation of active MT1-MMP and reduction of the 44-kDa form are also observed with synthetic MMP inhibitors consistent with the autocatalytic nature of this process (6). Thus, both TIMP-2 and synthetic MMP inhibitors regulate the nature of the MT1-MMP forms present in the cells. Here, we examined the lysates of BS-C-1 cells expressing MT1-MMP alone or with TIMP-4 for the profile of MT1-MMP forms by immunoblot anal-
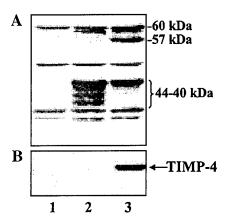

FIG. 3. Profile of MT1-MMP forms in the presence of TIMP-4. (A) BS-C-1 cells were infected–transfected to express MT1-MMP alone (lane 2) or with TIMP-4 (lane 3) as described in the legend to Fig. 2A. As control, some cells were infected–transfected with empty vector (lane 1). The cells lysates were resolved by reducing 10% SDS–PAGE followed by immunoblot analysis with anti-MT1-MMP antibodies and detection by ECL. (B) The same blot was reprobed with anti-TIMP-4 antibodies and developed by ECL.

ysis. As shown in Fig. 3, in the absence of TIMP-4, MT1-MMP is detected in its 60-, 57-, and 44–40-kDa forms (Fig. 3, lane 2) as previously reported (6). The 60-kDa protein represents pro-MT1-MMP starting at S^{24} (6). The 62-, 55-, and 37–35 kDa bands are nonspecific, as they are detected in infected cells transfected with empty vector (Fig. 3, lane 1). In the presence of TIMP-4 (Fig. 3, lane 3), there is a significant increase in the 57-kDa species of MT1-MMP and a reduction in the 44-40-kDa forms. Figure 3B shows the presence of TIMP-4 in the same lysates as detected with a specific anti-TIMP-4 antibody (lane 3) (21). Similar results were obtained with the *Timp2* ($-/-$) mutant cells (data not shown). From these results we conclude that, like TIMP-2, TIMP-4 inhibits the autocatalytic turnover of MT1-MMP and thus, TIMP-4 is likely to form a complex with the active form of MT1-MMP. This is further supported by the lack of detection of TIMP-4 in the supernatant of cells co-expressing MT1-MMP with the inhibitor as discussed above. Although not directly proven here, these results suggest that the lack of pro-MMP-2 activation in the presence of TIMP-4 is due to the inability of this inhibitor to generate a ternary complex with MT1-MMP and pro-MMP-2. Thus, while TIMP-4 binds to active MT1-MMP, the TIMP-4/MT1-MMP complex cannot act as a receptor for pro-MMP-2 and therefore activation does not ensue. The structural constrains that impede the formation of the ternary complex awaits elucidation of the crystal structure of the TIMP-4/MT1-MMP complex. In conclusion, the results of this study demonstrate a differential role for members of the TIMP family in the inhibition of MT1-MMP activity and in MT1-MMP-dependent pro-MMP-2 activation.